PREPARATION AND BIOLOGICAL PROPERTIES OF GLYCOSYLATED INSULIN

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1. Introduction

Since the discovery of hemoglobin A_{Ic} (HbA_{Ic}) [1] the sugar containing hemoglobins have attained appreciable interest both from the chemical and the pathophysiological point of view particularly in diabetes mellitus. In these hemoglobins the carbohydrate residues are non-enzymatically incorporated into the β-chains of hemoglobin AII (HbAII) via stable ketoamine linkages with the α-amino groups of the N-terminal valines. For the vast literature the reader is referred to some review articles covering the field [2,3]. More recently glucose and other sugars have been shown to react in vitro under physiological conditions with human HbAII to form the glycosylated hemoglobins HbA_{Ia+b} and HbA_{Ic} [4–6]. As it seemed likely to us that a similar type of glycosylation might occur with proteins other than hemoglobin we have studied the interaction of insulin with glucose and mannose. In the following we present data indicating that both hexoses are covalently incorporated into the insulin molecule upon incubation in vitro at 37°C. Furthermore it is shown that glucosylated insulin has reduced biological activity when assayed for stimulation of glucose oxidation, lipogenesis and antilipolysis in adipose tissue.

2. Materials and methods

Crystalline bovine insulin preparations from Serva (Heidelberg) Hoechst (Frankfurt/Main) and from Novo (Bagsvaerd) were used. D(+)-glucose and D(+)-mannose were from Merck (Darmstadt) D-[U-^{14}C]glucose, D-[1-^{3}H]mannose and D-[1-^{14}C]-glucose came from The Radiochemical Centre (Amersham). ATP, phosphoenolpyruvate, NADH, glycerokinase and pyruvate kinase were from Boehringer (Mannheim). Isoproterenol-hydrochloride was from Serva (Heidelberg). Bovine serum albumin came from Behringwerke, Marburg/Lahn. Collagenase was from Worthington, (Nutley, NJ), NCS from Searle (Heusenstamm), Thiocid from (Asid, Munich).

2.1. *Glycosylation of insulin*

To 0.1 ml of a 0.04% solution of insulin (Hoechst or Novo) in 0.002 N HCl, 0.8 ml Earle's medium [7] and 0.1 ml 40% (w/v) glucose or mannose dissolved in Earle's medium were added. After adjusting to pH 7.4 by gassing with carbogen the samples were incubated for 17 h at 37°C and stopped by freezing. The controls contained Earle's medium only.

2.2. *Insulin bioassays*

(a) The stimulation of [1-^{14}C]glucose oxidation using fat pads prepared from 200–250 g fed Sprague Dawley rats as described in [8] was measured according to [9] except that Krebs-Ringer phosphate buffer (pH 7.4) containing 1.3 mmol/l $CaCl_2$, 100 mg/dl of bovine serum albumin, and 100 mg/dl of [1-^{14}C]glucose corresponding to 5 μCi/dl was used.

(b) For the study of lipogenesis adipocytes from 100–140 g fed Sprague-Dawley rats were prepared according to [10] except that the buffer described in [11] fortified with 2.5 g/dl bovine serum albumin was used. Lipogenesis from D-[U-^{14}C]glucose was determined according to [12].

(c) Antilipolysis in adipocytes prepared as above was studied according to [13] except that 1 nmol/ml of isoproterenol was used. Glycerol was determined by the method in [14].

2.3. *Insulin radioimmunoassay*

This was conducted with the test kit from Novo.

3. Results

On incubation of insulin with labelled glucose or mannose, the hexose becomes linked to the hormone in a manner that indicates a covalent binding for the following reasons:

(i) ^{14}C-glucose or ^{3}H-mannose was not removed from insulin by extensive dialysis against a large excess of unlabelled sugar;

(ii) The trichloroacetic acid precipitate of an incubation mixture retained a constant amount of radioactivity even after several washings with 10% (w/v) trichloroacetic acid;

(iii) After electrophoresis of a dialyzed incubation mixture on SDS–polyacrylamide gels the distribution of radioactivity coincided with the insulin (fig.1).

The initial velocity of mannose incorporation was considerably faster than that of glucose (fig.2). The amount of hexose taken up by insulin proved to be a linear function of the sugar concentration in the incubation mixture when tested up to 220 mmol/l (fig.3). At this concentration 3.6 ± 0.39, (n = 16) glucose and 5.0 ± 0.43 (n = 10) mannose residues, respectively, were taken up per molecule of insulin when it was present at 1 U/ml. As shown in fig.4 glucosylation of insulin diminished the hormone's effect on [1-^{14}C]glucose oxidation by adipose tissue. At 25, 50 and 100 μU/ml of insulin the decrease in insulin stimulation amounted to 33%, 41% and 28%,

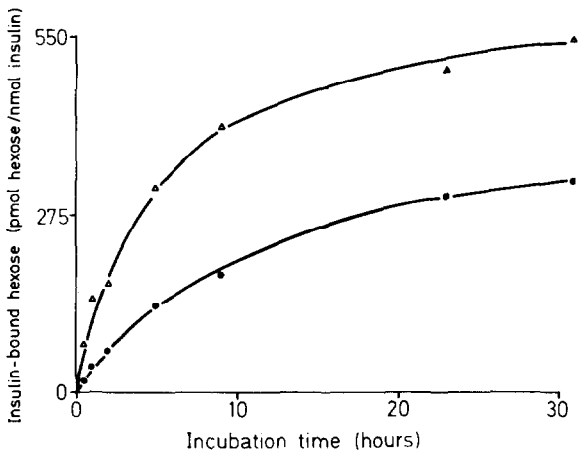

Fig.2. Formation of ^{14}C-glucose- and ^{3}H-mannose-insulin as a function of the incubation time. Insulin (Serva) 100 mg/dl in Earle's medium containing 0.01% Thiocid was incubated under shaking (pH 7.4) at 37°C with 44.4 mmol/l [^{14}C]glucose (•) or [^{3}H]mannose (△) at spec. radioact. 0.225 mCi/mmol for the times indicated, before 0.1 ml 3 mol/l trichloroacetic acid was added to 0.5 ml of the reaction mixture. After centrifugation the precipitate was washed 3 times with 1 ml 10% trichloroacetic acid dissolved by adding 0.5 ml NCS and 0.1 ml H_2O prior to neutralization with 0.02 ml 100% (w/v) acetic acid and counted for radioactivity in a liquid scintillation counter.

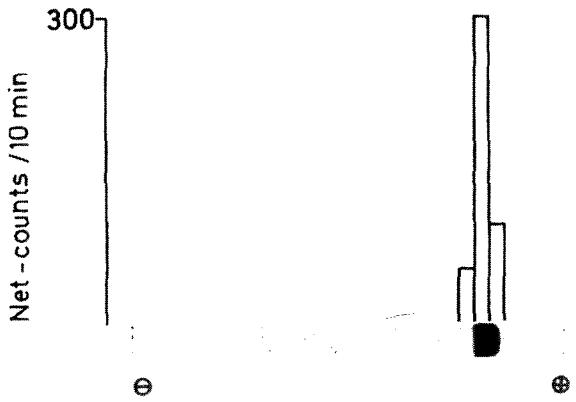

Fig.1. Analysis of ^{14}C-glucosylated insulin by SDS–polyacrylamide gel electrophoresis. Insulin (Hoechst) 3 mg in 1.5 ml Earle's medium [7] containing 0.5 mmol D-[U-^{14}C]glucose (corresponding to 150 μCi) and 0.01% Thiocid, was incubated in a shaking water bath at 37°C for 25 h. Before the plastic tubes (2 × 10 cm) were stoppered the pH was adjusted to 7.4 by gassing with carbogen. After extensive dialysis against neutral 0.9% NaCl at 4°C, SDS–gel electrophoresis was performed according to [18] using 0.02 ml the dialyzed reaction mixture corresponding to ~25 μg protein and 2300 cpm. Protein staining was accomplished with amido black 10B; the radioactivity in the slices of an unstained gel was determined in a Packard tricarb scintillation counter. The same results were obtained with labelled mannose instead of glucose.

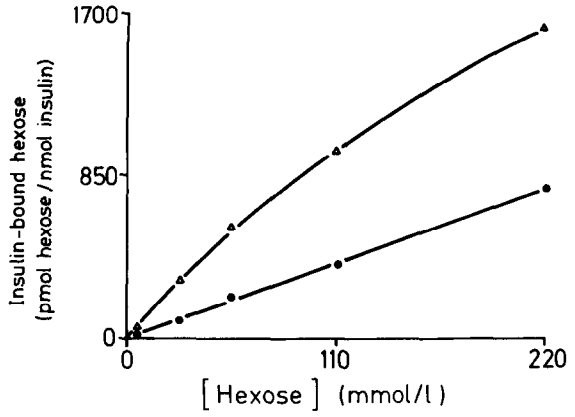

Fig.3. Effect of sugar concentration on ^{14}C-glucose- and ^{3}H-mannose-insulin formation. Insulin (Serva) 45 mg/dl in Earle's medium (pH 7.4) was incubated under shaking at 37°C for 3 h at different glucose (•) and mannose (△) concentrations at constant specific radioact. 0.09 mCi/mmol. Protein-bound radioactivity was determined as in the legend to fig.2.

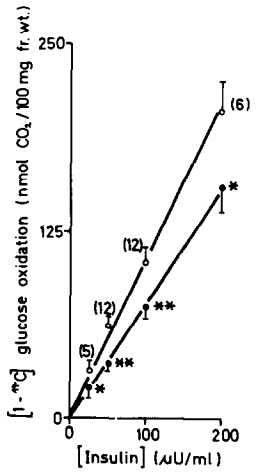

Fig.4. Effect of insulin and glucosylated insulin on glucose oxidation. Insulin was incubated in the presence (•) and absence (○) of glucose as in section 2.1 and diluted with the buffer used for incubation to give the final concentrations indicated. For further details see section 2.2(a). Mean values ± SEM for the numbers of different experiments in parentheses are given. Statistical significance was calculated by Student's t-test for paired data: $*p < 0.005$; $**p < 0.0005$.

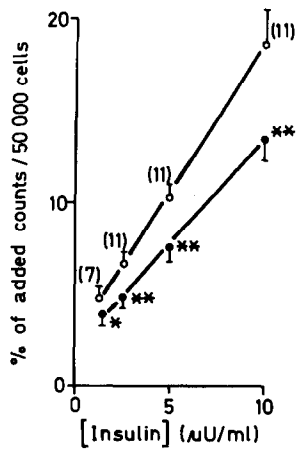

Fig.5. Effect of insulin and glucosylated insulin on lipogenesis. Insulin was incubated in the presence (•) and absence (○) of glucose as in section 2.1 and diluted with the buffer used for incubation to give the final concentrations indicated. For further details see section 2.2(b). Mean values ± SEM for the numbers of different experiments in parentheses are given. Statistical significance was calculated by Student's t-test for paired data: $*p < 0.005$; $**p < 0.0005$.

respectively. Also with respect to lipogenesis the response of adipocytes was significantly lower in the presence of the glucosylated insulin as compared to the native hormone (fig.5). Moreover the antilipolytic action of 12.5, 25, 50 and 100 μU/ml of insulin in 7–9 different experiments with isolated fat cells was significantly ($p < 0.0005$) decreased by glucosylation (range 9%–16%), although this effect compared with that on glucose oxidation and lipogenesis was relatively small.

Table 1
Immunologic property of glucosylated insulin

Sample	Insulin (μU/ml)
Human serum (HS)	11.7 ± 0.9
HS + 40 μU/ml glucosylated insulin	46.1 ± 2.7
HS + 40 μU/ml non-glucosylated insulin	46.0 ± 2.8

Glucosylated insulin prepared as in section 2.1 was added to human serum prior to the radioimmunoassay for insulin with the Novo test kit. Mean values ± SEM of 5 experiments are given

A different situation was met regarding the biological activity of the mannosylated insulin which, over 25–200 μU/ml (4–7 expt.) exhibited unchanged stimulation of glucose oxidation and an only marginal (< 10%) lowering of antilipolysis. In contrast to the biological activity, the immunological reactivity of insulin was not altered by glucosylation as the radioimmunoassay did not allow to differentiate between the native and the glucosylated insulin (table 1).

4. Discussion

To our knowledge the only report on glucose binding to insulin in aqueous solution is that in [15]. In equilibrium dialysis experiments these authors found that up to 8 molecules of glucose could be bound per molecule of insulin, however, they did neither consider the possibility of a covalent glucose incorporation into insulin, nor a change in the biological functions of the hormone. Insulin has also served as a model for studies of the Maillard reaction in food chemistry [16]. When stored in solid form at glucose concentrations of 430 mmol/l for 120 days at 37°C and 68% relative humidity on an average 3.7 glucose residues were bound to the hormone, a figure similar to that obtained by us under quite different conditions. Our preparations of glucosylated insulin resembled those in [16] also with respect to the alteration of biological activity, as the Maillard insulin had lost 20% of its potency to depress the blood glucose concentration in rabbits.

Insulin may now be added to hemoglobin and the lens crystallins [17] as another protein where covalent incorporation of sugar can occur non-enzymatically under physiological conditions leading to functional changes of the molecule. So far the only example indicating that such an interaction takes in fact place in vivo is the increase of the glycosylated hemoglobins in diabetic patients. Although the possibility exists, it remains to be established whether glycosylated insulin is also formed in vivo under pathological conditions.

Acknowledgements

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